CHEMICAL COMPARISON OF HIDES AFTER ENZYME-UNHAIRING AND LIME-UNHAIRING*

C. J. W. WIEGAND AND FRANCIS V. MORRISS

Midwest Research Institute

Kansas City, Missouri

and

WALLACE WINDUS

Eastern Regional Research Laboratory†

Philadelphia 18, Pennsylvania

ABSTRACT

The purpose of this research was to investigate chemical differences between enzyme-unhaired hides and lime-unhaired hides. Hide samples so prepared were analyzed for total nitrogen, amide nitrogen, and amine nitrogen. Comparisons were made of the N-terminal amino acids by end group analyses. Aspartic acid and serine were found to be terminal members in all samples, and glutamic acid was found in all samples except those unhaired by liming and by pulping.

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INTRODUCTION

Processes are now available which unhair hides by enzymatic action. However, the changes which take place in the hide during the depilation differ from those occurring in the conventional lime-sulfide processes. These differences are most evident from the quality of the leathers that can be produced.

The literature of leather technology contains many comparative studies of the effect of process variables which show that liming removes some constituents of the skin. Reports of experience with enzymatic unhairing indicate that these are not affected or removed by the liquors employed in the enzyme processes. Other evidence suggests that lime produces subtle changes in the collagen fibers of the hide that have a substantial influence upon the quality of the leather.

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This paper reports the results of a study undertaken to reveal any attack on the collagen by the process reagents during enzyme-unhairing and liming.

Representative procedures were employed to simulate plant practices. A companion paper (1) will present the results of electron microscopic examination and wide-angle X-ray studies of the materials reported.

EXPERIMENTAL METHODS

The back bend area of a 35-lb. brined and defleshed steerhide was divided into 18 approximately 6½" x 10" samples as shown in Fig. 1. Pairs of samples, one of each from the left and right side of the hide, were selected and processed together. The pairs of sample pieces averaged 224 g. (202 g. min. and 234 g. max.). The average green-salted weights of paired samples were employed for calculations throughout the processing. The distribution of the processing of the samples is also noted in Fig. 1.

The following treatments were given the pairs of samples:

Soaking

For enzyme unhairing—The 12 pieces totaling 2,767 g. were placed in 11 l. of water (4:1 soak) containing 6.5 g. Dowicide B.* They were stirred several times and allowed to soak for 24 hr. The container was held in a running-water bath at 74°F. The soaked pieces were transferred to fresh water, washed for 15 min. in running water, and drained of excess water.

For conventional unhairing—The six pieces totaling 1,295 g. were soaked in 6.5 l. of water (5:1 soak) for 24 hr. The container was placed in a water bath to maintain a temperature of 74°F. The soaked samples were transferred to fresh water, washed in running water for 1 hr., and drained of excess water.

Unhairing

*With Enzyme A-H.T. Proteolytic** (*Miles Laboratories, Inc.*).—The three pairs of samples (approximately 450 g. each pair) were placed in each of three 1-gal. jars containing 2,200 ml. water (5:1 float), 107 g. sodium chloride, 15 g. sodium borate, 1.1 g. Dowicide B, and 4.3 g. H.T. Proteolytic enzyme (561 NU/g). The jars were strapped to a mechanical shaker in a constant temperature cabinet (90°F.), shaken intermittently for 5 hr., and allowed to stand at 90°F. for an additional 18 hr. The pieces were removed from the enzyme solutions, scudded with a stout spatula to remove the hair, washed in running water for 30 min. and drained.

*With Enzyme B-M-zyme** (*Merck*).—The three pairs of samples (approximately 450 g. each pair) were treated the same as those above except that 11 ml. M-zyme (30,000 keratinase units/ml.) was used.

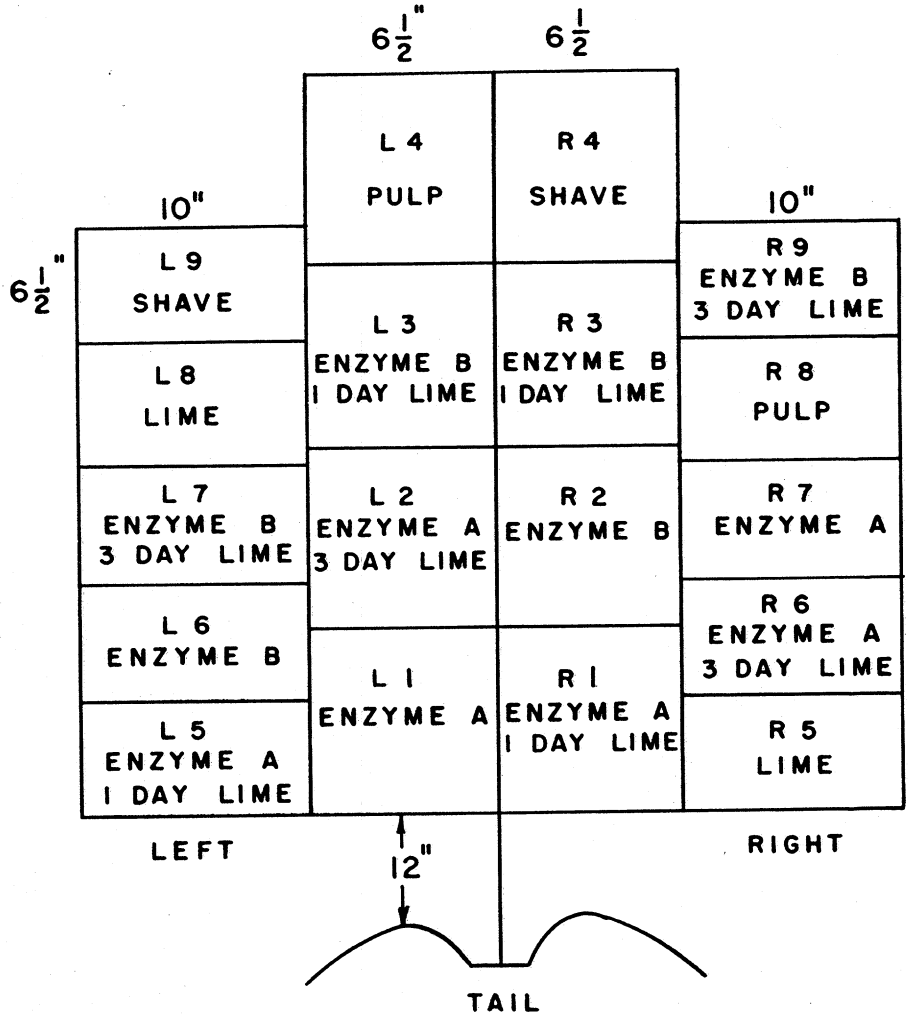

FIGURE 1.—Division of back bend area of steerhide and treatments applied to the samples.

By shaving.—The hair was removed from one pair of samples by shaving with a razor.

With lime-sodium sulfide.—Pairs of samples (approximately 450 g. each pair) were placed in 1-gal. jars containing 50 g. calcium hydroxide, 2.5 g. sodium sulfide (60%), and 2,500 ml. water (5:1 float) and were held in a constant temperature room (75 °F.) for either the 1- or 3-day period required by the experiment. On the first day the jars were shaken occasionally during the first 4 to 6 hr. On subsequent days they were shaken several times in the morning and afternoon. At the end of the specified liming period the hair

was removed by scudding. Each pair of samples was washed in running water for 15 min. and drained of excess water in preparation for bating.

By hair pulping.—The two pieces (408 g.) were placed in a solution of 12 g. sodium sulfide (60%) in 2,200 ml. water (5:1 float) contained in a 1-gal. jar and held at 76°F. for 24 hr., with occasional shaking during the first 8 hr. After residual hair was scraped off the samples, they were washed in running water for 15 min. and placed in a mixture of 40 g. calcium hydroxide in 2,000 ml. water. After standing at 76°F. for 24 hr., the samples were washed in running water for 15 min. and drained in preparation for bating.

Liming of enzyme-unhaired samples.—The effect of liming after enzyme-unhairing was studied. Paired samples unhaired with Enzymes A and B were limed for 1 day and for 3 days, respectively, using the condition outlined under the unhairing procedure with lime-sodium sulfide.

Bating.—The pairs of samples were placed in a mixture of 10 g. ammonium sulfate, 0.5 g. Oropon N*, and 2,500 ml. water (5:1 float) contained in a 1-gal. jar. The jar was strapped to a mechanical shaker and shaken vigorously. At the end of 2 hr. the hide samples were tested with phenolphthalein. In all samples a 3-hr. bating was necessary for deliming. The samples were removed from the bate and washed in running water.

Washing and drying.—After completion of the prescribed process treatments, each pair of samples was washed in running water for 1 hr., drained, and dehydrated by immersion for 24 hr. each in two successive changes of 2 l. of acetone. Residual acetone was removed under reduced pressure in a vacuum desiccator. The pieces were equilibrated in air for about 48 hr. and then cut into ¼″ cubes. From the approximate center of each piece, a 1″ x 2″ section was reserved for use in physical studies of the collagen fibers (1).

Analyses or analytical procedures.—Nitrogen analyses were made by the Kjeldahl method for total nitrogen, the Van Slyke method for amino nitrogen, and the method according to Mellon *et al.* (2) for amide nitrogen. Ether extraction of the samples showed less than 0.1% fats.

End group determinations were made by labeling and recovering the N-terminal amino acids after the method of Sanger (3). In preliminary studies conditions were determined so that the dinitrophenylation step allowed complete penetration of the hide samples. Penetration was confirmed by observing the color of the innermost fibers under the microscope. The quantity of reagents provided a generous excess of unreacted 2,4-dinitrofluorobenzene and bicarbonate at the end of the reaction period. These were completely removed during the wash cycles. The hydrolyses of labeled

material were run under nitrogen to prevent the oxidative side reactions of dinitrophenyl products. A test of the hydrolyzate after completion of the extraction did not show the presence of dinitrophenylated di- or polypeptides (4), nor were any of these found in the final ether extracts containing the dinitrophenylated amino acids.

From these preliminary studies the following procedure was developed for use in the studies:

The diced hide sample (25 g.) was placed in a 500-ml. Erlenmeyer flask containing 100 ml. ethanol, 6 g. sodium bicarbonate, 100 ml. water, and 10 g. 2,4-dinitrofluorobenzene. The flask was loosely stoppered and shaken for 25 hr. at room temperature. The solvent layer was removed, and the sample was washed by shaking 4 hr. with 200 ml. 50% ethanol. The wash solvent was replaced with fresh 50% ethanol, and shaking was continued 4 hr. Finally the ethanol was replaced with 200 ml. water and shaken overnight (16 hr.). The water was removed, and the sample was weighed and transferred to a 500-ml. round-bottom flask. The calculated quantity of water and hydrochloric acid to provide 250 ml. of 1:1 acid was added.

The samples were digested for 16 to 18 hr. at room temperature. The grain layer remained essentially intact, whereas the body of the hide had completely dissolved. The hydrolysis was completed by boiling the mixture at reflux temperature for 16 hr. under nitrogen. The reaction mixture was evaporated to dryness under vacuum in a rotary evaporator to remove excess hydrochloric acid. A solution of the dried product in 200 ml. water was neutralized and adjusted to pH 8 with sodium carbonate. Dinitroaniline was removed by three 50-ml. chloroform extractions. Dinitrophenol was removed by three 50-ml. chloroform extractions after the solution had been strongly acidified (pH 0.5) with hydrochloric acid. The dinitrophenyl amino acids were then extracted by three 50-ml. portions of ether. The solution was washed through acidified water, evaporated to a small volume, and divided into equal portions. One portion was evaporated to dryness to determine the quantity of solute, and the other was retained for paper chromatographic analysis following Mellon's (5) technique.

RESULTS AND DISCUSSION

The results of nitrogen determinations are shown in Table I, and these data have been used to prepare Table II, showing the fraction of the nitrogen present as amide and amino nitrogen. From these observations it is seen that all the processing operations result in increased nitrogen content. Removal of non-nitrogenous material, however, appears to be greatest by lime treatments after enzyme-unhairing. It is interesting that the unhairing with lime alone or with Enzyme B produced the least change in the nitrogen content.

TABLE I

ANALYSES OF PROCESSED SAMPLES

Sample No.*	Moisture %	Total Nitrogen† %	Ash %	Amino Nitrogen† %	Amide Nitrogen† %
L1	14.60	18.53	1.00	0.47	0.66
L2	14.87	18.37	1.12	0.44	0.55
L3	14.16	18.62	0.90	0.46	0.58
L4	14.52	18.35	0.92	0.45	0.60
L5	14.95	18.37	0.90	0.45	0.59
L6	15.27	18.04	1.47	0.49	0.62
L7	15.76	18.05	1.25	0.46	0.52
L8	15.02	18.05	1.23	0.42	0.51
L9	14.86	17.92	2.06	0.47	0.65
R1	17.72	18.59	0.89	0.42	0.61
R2	17.34	18.51	0.96	0.46	0.63
R3	17.54	18.91	0.56	0.46	0.60
R4	16.72	18.25	1.60	0.48	0.67
R5	17.67	18.48	1.23	0.45	0.57
R6	17.25	18.44	1.03	0.42	0.55
R7	17.16	18.45	0.59	0.44	0.64
R8	16.83	18.57	1.01	0.44	0.62
R9	16.65	18.72	1.18	0.43	0.53

*See Fig. 1 for treatments.
†Dry basis.

The changes in the ratios of the amino nitrogen and of the amide nitrogen brought about by the various process steps differ for each treatment. It is interesting that treatment with Enzyme B has no effect on the relative content of amino nitrogen, whereas all other treatments including that with Enzyme A produced an appreciable (7–11%) decrease in this factor. The changes in the amine and the amide nitrogen content of the hide proteins were of the same order of magnitude. Liming alone or lime treatments after either enzyme treatment produced the greatest change in amide nitrogen.

It has been demonstrated by Deasy (6) that relatively large samples of steerhide corium are required for the determination of the N-terminal amino acids of collagen by the Sanger method. She found serine, aspartic acid, and glycine to be N-terminal in two samples of steerhide corium.

Dinitroaniline, dinitrophenol, and other nitro compounds interfered in the paper chromatographic analyses of the solutions of dinitrophenyl amino acids. The large quantities of these encountered in the hydrolyzates of the labeled hide samples also interfered with the ultimate separation and isolation of the dinitrophenyl amino acids. The extraction techniques that were used in this study effectively removed the interfering materials to provide a final extract that contained essentially only dinitrophenyl amino acids. The large quanti-

ties of ϵ-dinitrophenyllysine derived from the protein remained in the aqueous phases during the extractions and did not interfere. No evidence was found for the presence of α-dinitrophenyl derivatives of lysine, arginine, or histidine in the aqueous phase.

TABLE II

EFFECT OF PROCESS TREATMENTS ON NITROGEN DISTRIBUTION IN HIDE

Sample	Treatment	% Total Nitrogen*	% Total N as Amino N	% Total N as Amide N
R4	Shave	18.55	2.61	3.66
L9		18.30	2.63	3.62
R5	Liming	18.71	2.42	3.09
L8		18.27	2.31	2.82
R8	Pulping	18.76	2.39	3.35
L4		18.52	2.45	3.26
R7	Enzyme A	18.56	2.36	3.45
L1		18.71	2.53	3.59
R1	Enzyme A	18.76	2.26	3.30
L5	plus 1 day liming	18.54	2.44	3.21
R6	Enzyme A	18.63	2.28	3.01
L2	plus 3 days liming	18.58	2.38	3.01
R2	Enzyme B	18.69	2.51	3.39
L6		18.31	2.73	3.42
R3	Enzyme B	19.02	2.44	3.20
L3	plus 1 day liming	18.79	2.44	3.14
R9	Enzyme B	18.94	2.30	2.83
L7	plus 3 days liming	18.28	2.54	2.86

*Corrected for moisture and ash.

The compilation of the dinitrophenyl amino acids found in hydrolyzates of the labeled hide samples of Table III shows some differences that may be of significance to the leather-processing problem. Aspartic acid was found to be a terminal acid in all the samples. The action of the Enzyme B appears to increase the quantity of terminal aspartic acid.

The dinitrophenylated material (Column *X*) was the major component recovered from the hydrolyzates of all the labeled samples. This material, upon further acid hydrolysis, was subsequently found to contain dinitro-

TABLE III

DNP AMINO ACIDS FOUND IN HYDROLYZATES OF DINITROPHENYLATED HIDE SAMPLES (Mg/g)

Sample	Treatment	Asp.	Glu.	X*	Gly.	Threo.	Meth.
R4	Shave	0.26	0.26	1.30	0	0.26	0
R8	Pulping	0.34	0	1.03	0.17	0	0
L8	Liming	0.24	0	1.92	0	0	0
R7	Enzyme A	0.34	0.17	1.18	0	0	0
R1	Enzyme A + 1-day lime	0.15	0.15	1.06	0	0	0
R6	Enzyme A + 3-day lime	0.21	0.21	1.46	0	0	0
L6	Enzyme B	0.45	0.22	1.34	0	0	0
L3	Enzyme B + 1-day lime	0.56	0.28	1.40	0	0	0
L7	Enzyme B + 3-day lime	0.27	0.27	1.36	0	0	0.27

*A mixture in which serine was identified.

phenyl serine and several unidentified colorless materials. The material accumulated at Rf=0.25 in butanol/water developer. It did not move in the benzene/acetic acid system. An authentic sample of dinitrophenyl-*L*-hydroxyproline showed the same Rf values. However, the yellow substance showed a maximum ultraviolet absorption of 360 mμ, indicating that it was not a hydroxyproline derivative. These are known to show a maximum absorption at 385 mμ (7). Further hydrolysis in hydrochloric acid solution produced a yellow substance of Rf=0.33 and Rf=0.08 in the above systems, corresponding to the recorded values for dinitrophenyl serine. Amino acids could not be detected in the hydrolyzate by paper chromatography. However, several bands were visible under ultraviolet light before and after treating the chromatograms with ninhydrin. These results suggest that serine is also a terminal group in the protein of the hides.

Only aspartic acid and serine were found as end groups in all samples after the various treatments. The quantity of dinitrophenyl aspartic acid recovered indicates that it is associated with less than 7,000–8,000 amino acid units comprising the protein terminated by it. This indicates a molecular weight of less than 1 million for the major proteins in the hide. These terminal groups may be associated with the differences in collagen fibrils revealed by electron microscopic studies of the samples (1).

Glutamic acid was found to be terminal in all samples except those unhaired by pulping and by liming. Apparently a glutamyl-terminated protein was dissolved and removed by the strong lime solutions. The detection of N-terminal glutamic acid in the enzyme-unhaired hides, even after liming, implies that another insoluble protein was involved. It is plausible to attribute the evidence to cleavage of a portion of the collagen by the enzymes at a glutamyl peptide bond to yield an alkali-insoluble collagen-derived protein. It is possible that cleavage of this type could contribute to the characteristics of leather prepared from enzyme-unhaired hide.

N-terminal threonine was found only in the unprocessed sample. The glycine found to be terminal in sample R8 could have been formed by the action of the sulfide-sharpened pulping treatment. Methionine, found to be N-terminal only in the 3-day limed sample after unhairing with Enzyme B, may result from a degraded protein.

CONCLUSIONS

Aspartic acid and serine were found as end groups in all samples following the various treatments. Glutamic acid was found terminal in all samples except those unhaired by lime and sulfide. The significance of changes in the hide composition brought about by unhairing techniques cannot be assessed without further experimental studies correlating the characteristics of leather with chemically detectable differences.

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